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INTRODUCTION

With Andrea Schubert, Ronald Koes and Francesca Quattrocchio

The anthocyanin biosynthetic pathway

Anthocyanins are water-soluble vacuolar pigments involved in the coloration of most tissues of higher plants, including flowers, fruits, leaves, seeds and roots. Anthocyanins provide a wide variety of colours, ranging from red-purple to blue-violet depending on their molecular structure, the presence of co-pigments and the pH of the solution (Tanaka et al., 2009). Anthocyanins consist of an anthocyanidin aglycone bound to sugar group(s), and accumulate in most plant cells as 3-glycosides. More than 400 different anthocyanins have been reported (Kong et al., 2003), which essentially differ by the type of substitutions on the three rings of the basic anthocyanidin skeleton. Since the absorption spectrum of these molecules depends on the pH of the medium in which they are dissolved, anthocyanins behave as pH indicators, turning from red to blue when the environment changes from acidic to basic.

The biosynthetic pathway responsible for the synthesis of the anthocyanins that colours flowers and fruits is a long-time favourite to study a wide variety of biological processes including the regulation of gene expression, RNA interference, transposition, and the intracellular transport of (secondary) metabolites. Due to the direct relation between gene activity and pigment accumulation, it has been ideal to unravel molecular mechanisms at the basis of the tissue specific gene expression and to study transcription factor networks. For these reasons, the anthocyanin pathway is one of the most studied pathways in plants, both biochemically and genetically.

Anthocyanins belong to a class of secondary metabolites termed flavonoids, which are all synthesized via the phenylpropanoid pathway. The biochemical route that produces flavonoids is well known in many species and many of the “structural” genes that encode the enzymes of the pathway have been cloned and characterized in a variety of species. Three plant species have been extensively used to elucidate the main steps of phenylpropanoid and flavonoid production by genetic approaches: petunia, snapdragon and maize. Although these three species differ in anthocyanin composition and pattern of accumulation, the majority of the reactions in anthocyanin synthesis are conserved in these, and other species (Koes et al., 1994).

Aromatic amino acids are channelled into phenylpropanoid biosynthesis through the enzyme phenylalanine ammonia lyase (PAL), which converts phenylalanine to cinnamic acid (Figure 1). The first reaction of the flavonoid pathway, catalyzed by chalcone synthase (CHS), involves the condensation of one molecule of 4-coumaroyl-CoA and three molecules of malonyl-CoA to yield tetrahydroxylchalcone. The petunia genome contains 12 different genes (or gene fragments) encoding CHS or CHS-like enzymes, but only two of them are expressed in floral tissues and contribute to anthocyanin synthesis (*chsA* and *chsJ*) (Koes et al., 1989). The product of this first reaction is isomerised by chalcone isomerase (CHI) to form naringenin, a colourless compound. In a further step, naringenin is converted into dihydrokaempferol by flavanone 3-hydroxylase (F3H) which was first isolated and cloned in petunia (Britsch et al., 1992).

Dihydrokaempferol can be hydroxylated either by flavonoid 3’hydroxylase (F3’H) or by flavonoid 3’5’ hydroxylase (F3’5’H) enzymes, determining the hydroxylation state of the anthocyanin produced in the plant. In petunia, two loci, *HYDROXYLATION AT THREE 1* (*Ht1*) and *Ht2,* control the activity of F3’H, which is required for the production of cyanidin-like anthocyanins, whereas two other loci, *HYDROXYLATION AT FIVE* (*Hf1*) and *Hf2* (Holton et al., 1993) control the F3’5’H activity, which leads

to the synthesis of delphinidin-like anthocyanins (Wiering, 1974). Three research groups have recently identified *F3'H* and *F3'5'H* genes in grape (Bogs et al., 2006; Castellarin et al., 2006; Jeong et al., 2006). The grapevine genome contains two copies of *F3'H* and several copies of *F3'5'H* clustered on a single chromosome (Castellarin et al., 2006). Expression of some of these genes in *P. hybrida ht1 hf1* mutants provided direct evidence that the encoded proteins indeed have F3'H and F3'5'H activity (Bogs et al., 2006).

The flavanones and dihydroflavonols produced by F3H, F3'H and F3'5'H are the precursors for several distinct branches of the flavonoid pathway, that each yield a distinct subclass of compounds, such as, for example, flavonols and anthocyanins. The enzyme dihydroflavonol 4-reductase (DFR) catalyzes the first step of the pathway that yields proanthocyanindins, (also known as condensed tannins) and anthocyanins. DFR catalyzes the reduction at position 4 of the C-ring to produce the colourless leucoanthocyanidins (Stafford and Lester, 1982). In maize and snapdragon *DFR* genes were cloned by trasposon tagging and it has been shown that mutations in the *a1* locus of maize eliminate anthocyanins from the aleurone of the kernel (Reddy et al., 1987) and mutations in the *pallida* locus of snapdragon produce colourless or partially coloured flowers (Almeida et al., 1989).

Subsequently, the enzyme leucoanthocyanidin dioxygenase (LDOX), also called anthocyanin synthase (ANS) (Heller and Forkmann, 1988), oxygenates leucoanthocyanidins to produce anthocyanins. This enzyme is responsible for the synthesis of the coloured anthocyanins that can be directly stored into the vacuole or, alternatively, additional decorations of the A, B or C rings may follow to yield a more substituted molecule. The anthocyanidins can be 3-glycosylated to stabilize their colour before the subsequent decoration steps.

The glycosylation of anthocyanins is catalyzed by UDP glucose transferases: flavonoid 3-*O* glucosyltransferase and 5-*O*-glucosyltransferase (Jonsson et al., 1984c). In some species, as petunia and snapdragon, the glycosylated anthocyanins are further modified via rhamnosylation to produce an anthocyanidin 3-rutinoside. In other plants, *O*-glycosylation of anthocyanindins takes place using as sugar donors UDP-galactose, UDP-rhamnose, UDP-xylose, UDP-glucuronic acid or UDP-arabinose (Springob et al., 2003). In petunia, the gene encoding UDP rhamnose: anthocyanin 3-glucoside rhamnosyltransferase (3RT) has been isolated by genetic approaches and was found to identical to the *RHAMNOSYLATION AT THREE* (*RT*) locus (Brugliera et al., 1994; Kroon et al., 1994). In grape, the genotypes which do not express genes for the initial glycosylation of anthocyanidins do not accumulate any anthocyanin in their berries, although the other enzymes for anthocyanidin biosynthetic pathway are present (Boss et al., 1996), suggesting that in the berries anthocyanidins are unstable. In grape, the enzyme UFGT (flavonoid glycosyltransferase) (Ford et al., 1998) glycosylates anthocyanins at the C3 position and its expression pattern follows the anthocyanin accumulation and is specific for pigmented tissues. This enzyme has high substrate specificity; indeed kinetic analysis has shown the preference for anthocyanin substrates instead of flavonols. In non- vinifera Vitis species, the glycosylation occurs at the C3 and also at the C5 position to form anthocyanin 3, 5-O-diglucosides (Mazza, 1995).

The enzymatic steps of the biosynthetic pathway that give the "final touch"

Additional modifications of the anthocyanin by methylation and acylation, decorate the synthesized molecules and help to stabilize these pigments and adjust the colour, and provide the end products of the pathway that are sequestered and stably accumulate in the vacuole of the cell. Little is known about the last steps of the anthocyanin biosynthetic pathway that are responsible for subtle differences in colour, which can be due to a multitude of possible decorations of the molecules. These last steps of the pathway involve enzymes like methyltransferases and acyltransferases. These enzymes occur in the

cytosol and are thought to be important to adjust the colour and stability of the pigment molecule, prior to their transport into the vacuole (Springob et al., 2003).

Methylated and acylated anthocyanins are very common in nature. As an example, in *Vitis vinifera* berries the concentration of methylated and acylated anthocyanins can be very high depending on the genotypes. In Barbera, one of the cultivar that we studied in this thesis, the di-methylated anthocyanin malvidin accounts for 36,6% of the total content of anthocyanins of 2230 mg per kg of berry, while the mono-methylated peonidin accounts for 5,46% (Mattivi et al., 2006). Acylated anthocyanins can also be abundant in the grape berry: in Barbera they account for 14% of total amount of anthocyanins, and these values can be higher in other genotypes. These differences in anthocyanin composition among grape varieties result from differences in the activity of the enzymes involved in the last steps of the anthocyanin biosynthetic pathway and play a fundamental role in defining the quality of wine.

Only in *Petunia hybrida* a few studies have reported the genetic control of methylation and other late steps of anthocyanin biosynthesis. Early genetic studies identified two loci controlling 3'methylation, *MT1*and *MT2,* and two loci controlling 5' methylation, *MF1* and *MF2* (Wiering, 1974). Also the *GF* locus has been identified as probably involved in the control of acylation (Jonsson, et al., 1984b), even though no other information is available in literature about this gene in petunia.

The genome of Arabidopsis contains at least 17 genes that encode a (putative) O-methyltransferase. The predicted proteins all contain the AdoMet binding domain while the substrate binding domain changes in relation of the substrate specificity, indicating that they are involved in different cellular process (Kim et al., 2005). Arabidopsis can produce anthocyanins in vegetative tissues (but not in the flowers) and the major representative is cyanidin (Bloor and Abrahams, 2002), which is an anthocyanidin that lacks methyl groups.

The first report of a grape methyltransferase (*AOMT)* involved in the methylation of anthocyanins is rather recent (Hugueney et al., 2009). This gene was shown to encode an enzyme that is able to methylate *in vitro* both 3' and 5' positions of the B-ring of anthocyanin molecules. In Chapter 3 we report the cloning and characterisation of three genes involved in the methylation of anthocyanin in petunia flowers and show, by complementation of petunia methylation mutants, that the grape VvAOMT can efficiently methylate anthocyanins *in vivo* which provides the final demonstration of the function of this gene *in vivo*.

Another late step in biosynthesis of anthocyanins involves the enzymes anthocyanin acyltransferases (AAT). These enzymes have been isolated and characterized only in a few species including *Gentiana triflora* (Fujiwara et al., 1998), *Perilla frutescens* (Yonekura-Sakakibara et al., 2000), *Salvia splendens* (Suzuki et al., 2001) and recently also *Arabidopsis thaliana* (Luo et al., 2007). AATs have strong substrate specificity for both the anthocyanin acceptors and the acyl group donors *in vitro*. On the basis of their preference for the acyl donor, acyltransferases can be divided in two groups (Mazza, 1995): those responsible for the aromatic acylation of anthocyanins, resulting in more stable and bluer pigment molecules due to intramolecular stacking of anthocyanins with polyphenols (Yonekura-Sakakibara et al., 2009), and those catalyzing aliphatic acylation, such as malonylation, which protects glycosides from enzymatic degradation (Suzuki et al., 2002), stabilizes anthocyanin structures (Saito et al., 1988; Suzuki et al., 2002), and facilitates uptake into the vacuole (Wolfgang and Seitz, 1987). Anthocyanin molecules that are not acylated can be easily and quickly decolorized in neutral or just weakly acidic aqueous solutions. However, these observations describe the behaviour of anthocyanins *in vitro* and that does not seem to reflect the situation *in vivo*. Indeed, petunia *gf* mutants accumulate non acylated anthocyanins in the acidic environment of the central vacuole of the epidermal

cells and they show strongly pigmented petals, meaning that the pigments are stable in these conditions.

In *Vitis vinifera*, as well in *Petunia hybrida,* the genes involved in the acylation of anthocyanins have not yet been identified, although the presence of acylated anthocyanins has been reported in both species. In this thesis we describe the characterization of anthocyanin acyltransferases in petunia and in grape (Chapter 4).

Anthocyanins are finally accumulated into the vacuole. So far the only described anthocyanin transporter is an MRP -type ABC transporter of maize (Goodman et al., 2004). ABC transporters of the MRP class accept glutathionylated substrates and their role in flavonoid transport was first suggested in 1995 when mutation of the maize GST Bronze-2 (BZ2) was shown to cause accumulation of anthocyanin in the cytosol of aleuron cells (Marrs et al., 1995). Mutations affecting a specific GST protein (AN9) block anthocyanin accumulation also in petunia (Alfenito et al., 1998). However, experiments trying to show that these GSTs can mediate the glutathionation of anthocyanins were not convincing. Therefore the possibility is still open that flavonoids do not get glutathionylated and the GST might function just as docking platform to the anthocyanin transporter, or as escorting factor for anthocyanins to the vacuole avoiding oxidation during the short time that they are present in the cytoplasm (Mueller et al., 2000). In Arabidopsis the *tt19* mutants (also encoding for a GST) show alterations in anthocyanin accumulation in shoots and in PA accumulation in seeds. TT19 is an AN9 homolog and complementation of *tt19* with petunia AN9 restores anthocyanidin but not PA accumulation (Kitamura et al., 2004). This suggests slighly different substrate specificity for AN9 and TT19 although it cannot be excluded that the activity of the 35S promoter used for this complementation study is not high enough in the cell types where TT19 needs to be expressed.

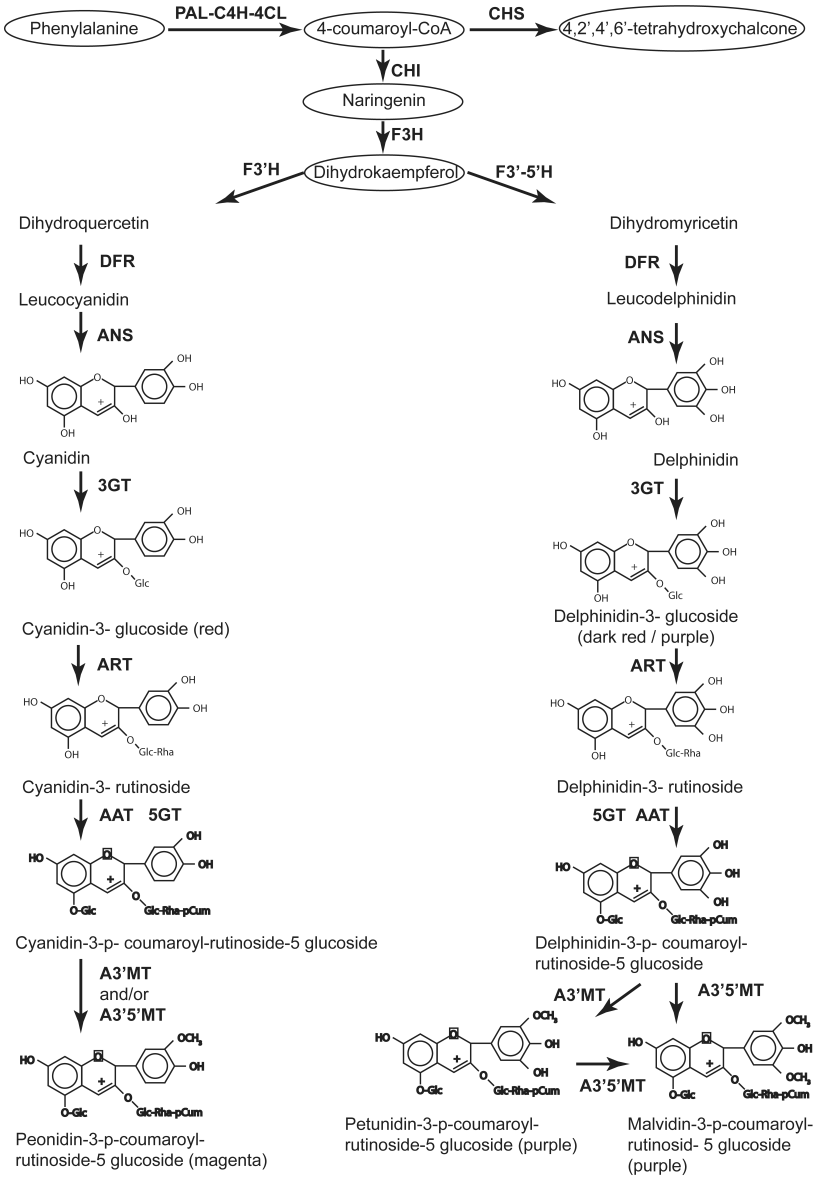
The Arabidopsis mutant *tt12* lacks proanthocyanidins in the seed coat and therefore displays pale seeds. The gene *TT12* encodes a transporter belonging to the MATE family which localizes in the tonoplast of seed coat cells and that accumulates tannins. The TT12 MATE transporter is involved in the transport of catechins to the vacuole of specialized cells, where polymerization can take place and tannins are finally stored (Debeaujon et al., 2001; Marinova et al., 2007).

Transport of anthocyanins into the vacuole has also been addressed in grapevine. A GST gene was found up regulated in coloured berries (Ageorges et al., 2006) and a GST and two MATE proteins (Cutanda-Perez et al., 2009) are activated in plants overexpressing the *VvmybA1* regulator of anthocyanin biosynthesis. GFP fusions of the two MATE transporters were shown to localize in the tonoplast when transformed into grape hairy roots and the recombinant proteins are able to transport *in vitro* acylated anthocyanins (Gomez et al., 2009). However the *in vivo* function of these proteins has not yet been demonstrated and therefore their involvement in anthocyanin transport remains hypothetical.

Control of vacuolar pH in cells accumulating anthocyanins

Anthocyanins, differ from other plant pigments, such as green chlorophylls, yellow and orange carotenoids and purple betalains, in their ability to exhibit a much wider variety of colours (Fukada-Tanaka et al., 2000). All six major anthocyanins (cyanidin, delphinidin, petunidin, malvidin, peonidin, pelargonidin) change their colour depending on the pH of the medium in which they are dissolved, resulting in a range of different displayed shades. *In vitro*, these molecules display a red colour in acidic solutions, a purple colour in neutral solutions or a blue colour in alkaline solutions (Tanaka et al., 2008).

Each of these colours can again give rise to a range of different hues when colourless co-pigments (such as flavones and flavonols) and/or metal ions are present. Since anthocyanins are sequestered in the vacuole, the pH of the vacuolar lumen influences the colour displayed by anthocyanins. The pH of this compartment is regulated by vacuolar ATPase (V-ATPase) and



pyrophosphatase (PPase) proton pumps (Gaxiola et al., 2002; Nishi and Forgac, 2002) in the tonoplast of plant cells. The V-ATPases are ancient enzymes with a well conserved structure that strongly resembles the structure of bacterial F-ATPase pumps and acidify a wide range of different organelles, using ATP to pump protons from the cytoplasm to the their lumen. This proton pumping system is composed of several subunits with two functional parts, V_1 which contains the catalytic site, and $V_{0,}$ which is a channel for protons.

The V-PPase is another proton pump able to acidify endomembrane compartments by using PPi (pyrophosphate) as energy donor. This pump is much simpler than the V-ATPase, as it consists of one single polypeptide strongly conserved among plants (similarity at amino acid sequence level 80-91%). These two pumps together control the pH of the vacuolar lumen and the lumen of several other organelles and compartments, and provide a membrane potential that drives the transport of different xenobiotics and plant metabolites by secondary transporters (Maeshima, 2001).

Figure 1. Schematic representation of the flavonoid biosynthesis pathways in *P. hybrida* petals**.** Enzymes involved in the pathway have been indicated as follows: PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4 hydroxylase; 4CL, 4-coumarate:CoA ligase; CHS, chalcone synthase; CHI, chalcone-flavanone isomerase;

F3H, flavanone 3-hydroxylase; F3'H, flavonoid 3'-hydroxylase; F3'5'H, flavonoid 3',5'-hydroxylase; DFR, dihydroflavonol4-reductase; ANS, anthocyanidin synthase; 3GT, UDP-glucose:anthocyanidin 3-glucosyltransferase; 3RT, UDP-rhamnose:anthocyanidin 3-glucoside rhamnosyltransferase; 5GT, UDP-glucose:anthocyanin 5-glucosyltransferase. Genetic loci controlling the pathway: *GF*, *GLUCOSYLATION AT FIVE'*, is required for the glucosylation in the 5 position, but the gene is not yet known (see next chapter) *MT1* and *MT2, l*oci responsible for 3' methylation (Jonsson et al., 1984a); *MF1* and *MF2* loci responsible for 3', 5' methylation. Flavonoid compounds: DHK, dihydrokaempferol; DHQ, dihydroquercetin; DHM, dihydromyricetin.

The characterization of flower colour mutants of Ipomea and petunia revealed several other tonoplast-localized transporters that control the acidity of the vacuoles where anthocyanins are stored. In morning glory (*Ipomea nil*) the petal colour changes from purple to blue when the flower bud opens, which is accompanied by a change in vacuolar pH from 6.6 to 7.7 (Yoshida et al., 1995). The recessive mutant *purple* is unable to increase its vacuolar pH to develop the blue petal colour. The *PURPLE* gene encodes a vacuolar $Na^+(K^+)/H^+$ exchanger which, at bud opening, consumes the proton gradient across the tonoplast resulting in the alkalinization of the vacuolar lumen (Fukada-Tanaka et al., 2000).

In *Petunia hybrida* petals the vacuolar lumen remains acidic and wild type lines bear red coloured flowers. Mutations in one of a set of seven loci, *pH1* to *pH7,* result in an increase of pH of the petal homogenate and a blue flower phenotype. This indicates that these loci are involved in the building and/or maintenance of an acidic vacuolar pH (de Vlaming et al., 1983; van Houwelingen et al., 1998).

One of these genes, *PH5*, has been shown to encode a P_{3A}-H^+-ATPase that resides in the vacuolar membrane and is required for the acidification of the vacuolar lumen (Verweij et al., 2008b). PH5 activity requires in petunia the presence of another P-ATPase (belonging to the P_{3B} subfamily) which is encoded by the *PH1* locus (Faraco, 2011).

In *Vitis vinifera* little is known about the regulation of vacuolar pH in tissues that accumulate anthocyanins. The low vacuolar pH in grape berries is supported by high concentrations (up to 50 mM total) of three organic acids: tartaric, malic, and citric acid. The pH of the pulp changes from 2.2 in the first stages of maturation, to 3.2 at the stages of grape harvest. Berry grape acidity is a parameter of major technological importance in winemaking and is routinely measured in the must, which is essentially vacuolar sap. In grape berries vacuolar pH can be very different in distinct cultivars and also depends on environmental conditions. In Chapter 5 of this thesis, we present the characterisation of the *PH1* and *PH5* genes from grape and rose and the function of these proteins is demonstrated by complementation of the corresponding petunia mutants. Although we cannot be sure of the direct involvement of these two pumps in the acidification of the vacuolar lumen in berry tissues, the correlation of their expression with anthocyanin deposition supports a role for PH1 and PH5 in the display of colour.

Anthocyanins: from plant to health

Anthocyanin production has been intensively studied in relation to its applications in the flower and in the food industries. The production of new flower colours in economically interesting species, results in new products which, depending on the species, can conquer a consistent portion of the market. In grapevine, anthocyanin research is spurred by the importance of colour in premium red wines. Furthermore the interest in anthocyanins and in their biochemistry and genetics has explosively grown in the last years because of the findings related to the effect of these compounds on human health.

Numerous recent studies indicate that flavonoids through the intake of anthocyanin-rich foods reduce the incidence of cardiovascular disease, cancer, hyperlipidemias and other chronic diseases. Martin and coworkers (Butelli et al., 2008) have shown that a diet containing high levels of anthocyanins can significantly increase the life span of cancer-susceptible *Trp53*$^{-/-}$ (transformation related protein 53*)* knockout mice. They have produced a purple tomato enriched for the content of delphinidin-like

anthocyanins derivatives, by constitutively expressing two transcription factors of snapdragon. Since it is known that anthocyanins have antioxidant effects, they fed *Trp53* $^{-/-}$ knockout mice with a diet supplemented with 10% of powder from purple tomato or red tomato (control). They found that mice treated with transgenic purple tomato had a significantly longer average life span compared with the control.

Anthocyanins are not only involved in cancer prevention but they can also improve the visual functions. Cyanidin 3-glucoside or rutinoside has been demonstrated to stimulate the regeneration of rhodopsin, which is a pigment of the retina responsible for the formation of photoreceptor cells and the perception of light (Matsumoto et al., 2003). Rhodopsin is a member of the G-protein coupled receptor family that undergoes a series of reactions to phototransduce the signal. In summary, several studies have shown that anthocyanins display a wide range of biological activities in animals with antioxidant (Wang et al., 1997; Fukumoto and Mazza, 2000), anti-inflammatory (Wang and Mazza, 2002; Youdim et al., 2002), antimicrobial (Pisha and Pezzuto, 1994), and anticarcinogenic activities (Kamei et al., 1995; Kang et al., 2003) and can improve of vision (Mercier et al., 1965; Matsumoto et al., 2003), induce apoptosis (Katsub. et al., 2003), and have neuroprotective effects (Youdim et al., 2000; Galli et al., 2002). In addition, anthocyanins display a variety of other effects on blood vessels (Andriambeloson et al., 1998; Martin et al., 2003) and platelets (Morazzoni and Magistretti, 1990; Demrow et al., 1995) that may reduce the risk of coronary heart disease (Renaud and de Lorgeril, 1992). Recent reports have shown that the antioxidant activity of anthocyanins decreases with the glycosilation of these molecules and it improves with an increase of hydroxyl groups. This property is demonstrated in several *in vitro* analyses. Pelargonidin, cyanidin, delphinidin, peonidin, malvidin, malvidin 3-glucoside, and malvidin 3, 5-diglucosides were found to have strong inhibitory effects on NO (nitric oxide) production and this effect is comparable with quercetin antioxidant and anti-inflammatory effects. Moreover, thanks to the presence of phenolic compounds in grape, red wine has a moderate antioxidant capacity, which decreases coronary heart disease risk in case of moderate consumption. The mechanisms responsible for this risk reduction include decreased platelet coagulation (Elwood et al., 1991; Renaud et al., 1992) and increased circulating high-density lipoprotein which binds cholesterol.

Recent studies have shown some molecular mechanisms of cancer prevention by anthocyanins (Hou De-Xing et al., 2004). These molecules seem to have effect on the apoptotic induction in cancer cells through reactive oxygen species or by inhibiting TPA (12-O-tetradecanoylphorbol-13-acetate), known to induce the AP-1 transcription factor which is involved in promoting carcinogenesis. The possibility to understand the molecular mechanism of action of anthocyanins, when introduced in the body with the diet, is the first step to develop a strategy for their use in drugs.

The studies published so far have shown beneficial effects of anthocyanins on human health, but definitive proof can be obtained only after long term trials. In the meantime the connection between anthocyanins and benefits on human health should include a more complete knowledge of the identity of anthocyanin metabolites and their tissue distribution using molecular, cell biology, and epidemiological studies.

Anthocyanins are widely used as food additives and they became very popular as “nutriceuticals” for their proposed positive effects on health. At the moment it is reasonable to expect that their use is going to increase and to become more targeted to the prevention of specific pathologies. Also flavonols are widespread in foods; the most prominent are quercetin and kaempferol. However, the concentration of flavonoids in general is very low in most consumed crops: e.g. flavonols of different classes are present at a concentration of 15-30 mg/kg fresh weight in broccoli, onions, kale, leeks, apples and blueberries (Hertog et al., 1992; Manach et al., 2004). Flavan 3-ols are present in many fruits as grape, teas, cocoa and chocolate. Anthocyanins, in particular, are most abundant in grape berries, certain

varieties of cereals, and some leafy and root vegetables such as cabbage, beans, onions, and radishes. The bioavailability (calculated as the fraction of the consumed ingredient that appears in the blood circulation) of polyphenols and flavonoids varies among the different subclasses of molecules. Flavonoids or flavonoid metabolites that reach the colon may be further metabolized by bacterial enzymes and absorbed. Colonic degradation by the microflora is extensive for procyanidins, the flavonol quercetin and flavan-3-ols. The flavonoids that are absorbed sufficiently to exert a possible effect on cardiovascular parameters *in vivo* include isoflavones, flavonols, flavanones and the flavan-3-ols (Manach et al., 2005). Due to the low presence of anthocyanins and other flavonoids in the natural products that are part of our diet and due to their limited absorption by the digestive system, much research is on going to improve the content of these compounds in food via breeding as well as transgenic approaches. Also the production of flavonoids in fermentors where plant cells can work as "mini green factories" is being explored.

Outline of the thesis

In this thesis I present an analysis of several genes that are involved in the last steps of anthocyanins production and I performed a comparative study with the aim to identify homologous genes in different species as Arabidopsis, rose, grape, carnation and petunia. For this purpose I have extensively explored the advantages of using a well established model system, like Petunia, to demonstrate the *in vivo* function of genes originating from different species. Petunia is a convenient system for the characterisation of genes involved in anthocyanin accumulation thanks to the collection of mutants affecting (almost) each step of the biosynthesis, transport and display of these pigments.

In **chapter 2**, I describe the cloning and characterization of a *Vitis vinifera* methyltransferase (*VvAOMT),* involved in methylation of anthocyanins in two cultivars (Syrah and Nebbiolo) which differ for the amount and type of accumulated anthocyanins. Enzymatic assays defined the enzyme properties, such as pH optimum, Km, sensitivity to metal inhibitors, and demonstrated the high specificity of this AOMT for anthocyanins substrates. Transformation of *At*PAP-expressing tobacco confirmed *in vivo* the function of the gene.

In **chapter 3**, I describe the isolation of three genes, *MT1*, *MF1* and *MF2*, that control the methylation of anthocyanins in *Petunia hybrida* flowers*,* and provide extensive genetic and biochemical evidence that they encode anthocyanin methyltransferases. The complementation of these mutants with the newly isolated genes represents the ultimate demonstration of the function of the petunia methyltransferases *in vivo*. I also expressed the *Vitis vinifera* methyltransferase, described in the previous chapter, in the same petunia mutants. The results of these transgenic experiments show that anthocyanins methyltransferases display, in spite of their high sequence conservation, remarkable differences in substrate specificity.

In the **chapter 4**, I describe the isolation of a novel anthocyanin acyltransferase that is encoded by the *GF* locus of *Petunia hybrida* (*PhDIFc*) and two candidate anthocyanin acyltransferases from *Vitis vinifera* (*VvAAT1* and *VvAAT2*). I analyzed a gene, *DIFc*, that was known to be activated by transcription factors of the anthocyanin pathway, but of which the function was not known. The *GF* locus of petunia was long suspected to encode an anthocyanin acyltransferase and we found lesions in the coding region of *PhDIFc* in *gf* mutants. We transformed a *35S:PhDIFc* construct into two *gf* petunia lines and the phenotype complementation confirmed that *PhDIFc* is encoded by the *GF* locus. The analyses of the anthocyanin contents in the transgenic flowers by HPLC have shown the accumulation of acylated product, demonstrating the acyltransferase activity of the PhDIFc/GF enzyme. We also transformed the same petunia *gf* lines with *35S:VvAAT1* and *35S:VvAAT2* and the results are on the way. From the phylogenetic analysis of a large number of acyltransferase proteins, it appears that these

three new anthocyanin acyltransferases are the founders of a new group of proteins involved in anthocyanins acylation.

Chapter 5 focuses on the regulation of pH in the vacuolar lumen of anthocyanin accumulating cells. The petunia tonoplast pumps *PH1* and *PH5* have been shown to be responsible for the acidification of this compartment in epidermal petal cells. To find out whether the PH1-PH5 pathway for vacuolar acidification is specific for petunia or is active in pigmented flowers of other species and possibly also fruits, we isolated homologs of *PH1* and *PH5* from Arabidopsis, grape, carnation and rose. By complementation of petunia *ph1* and *ph5* mutants respectively, we demonstrated that the function of these genes is widely conserved among different species. Interestingly, while *PH5* homologs are present in all species, the distribution of PH1 homologs seems to be much narrower as several species do not have one. We discuss the possible contribution of PH1 and PH5 to different aspects of anthocyanin and tannin accumulation in different plant species.

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